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IMMUNOLOGICAL AND TOXIC DIFFERENCES BETWEEN MOUSE-VIRULENT AND MOUSE-AVIRULENT *CANDIDA ALBICANS*¹

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ABSTRACT

ISENBERG, HENRY D. (Long Island Jewish Hospital, New Hyde Park, N.Y.), JONA ALLERHAND, JAMES I. BERKMAN, AND DOROTHY GOLDBERG. Immunological and toxic differences between mouse-virulent and mouse-avirulent *Candida albicans*. J. Bacteriol. **86**:1010-1018. 1963.—The differences between mouse-avirulent and -virulent strains of *Candida albicans* reside at least in part in cell surface materials which can be extracted with solvents such as ethanol-ethyl ether and phenol. These extracts are complex haptens which behave like endotoxins in mice and rabbits. Antibodies produced against intact and extracted cells show some strain specificity in agglutination and precipitin reactions, but underline primarily the differences between the virulent and avirulent variants. The chemical constitution of the extracted complex haptens suggests that the toxic or virulent principles are polysaccharide in nature and that the avirulent strain substitutes fats and lipids for some polysaccharides on their cell surface.

Investigations of *Candida albicans* in this laboratory have been directed primarily at the microbial and host factors which effect the change from amphibiont (Rosebury, 1961) to pathogen. Initially, this viewpoint led to a study of the ecological relationships of the yeast and those microorganisms it would encounter most often when introduced by chance into or on a human host (Isenberg et al., 1960). It was learned that under these circumstances the representatives of the resident bacterial species

exerted no effect on *C. albicans*, curtailed its proliferation, or stimulated its growth. This selective influence was dependent in part on atmospheric conditions. Thus, a microaerophilic, carbon dioxide-rich, gaseous environment was most favorable for the yeast, whereas aerobic conditions exceeded the strictly anaerobic ones in enhancing the inhibitory potential of the bacteria. In addition, it was learned that the inhibition of *Candida* by bacteria which did not elaborate specific antifungal antibiotics was due to crystalloid end products of their metabolism, primarily monocarboxylic unbranched organic acids of intermediate chain length, certain amines arising from the decarboxylation of amino acids, and sulfides (Isenberg, Berkman, and Carito, 1961). Unpublished findings indicated that the stimulatory compounds are incompletely oxidized intermediates of the metabolism of those bacteria which displayed this activity. Therefore, the ability of *C. albicans* to colonize any surface of the host is dependent upon the sum total of the activities of all the various components of the entire microflora present at any specific moment. To begin an approximation of the host-yeast interactions, the next logical sequence in this study of factors leading to overt monilial disease, the experiments reported here were undertaken.

MATERIALS AND METHODS

Microorganisms. The yeasts used were three variants of *C. albicans*: the avirulent laboratory strain 1529, designated as A in this report, and the mouse virulent strains B311 and 207, obtained through the courtesy of H. Hasenclever, National Institutes of Health, Bethesda, Md., and designated as V₁ and V₂, respectively. The organisms were maintained on Mycophil Agar (BBL) slants. For the production of cell material, the organisms

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were spread onto Mycophil Agar contained in large presterilized aluminum pans covered with heavy-duty aluminum foil. They were incubated at 37 C for 48 hr, harvested with minimal quantities of sterile 0.9% NaCl, transferred to 500-ml centrifuge bottles, centrifuged, and washed thrice with 0.9% NaCl. These cells served as starting materials for the various preparations.

Animals. Rabbits weighing 2 to 3 kg were used for the production of antibodies. Young adult male Swiss albino mice were used for the assessment of virulence of the yeast strains, the toxicity and skin-necrotizing properties of some of the extracts, and the ability of these fractions to protect against challenge with homologous and heterologous strains.

Extraction procedures. The procedures which produced the most suitable materials for the purpose of this study are summarized in Fig. 1 and consisted of (i) an ethanol-ethyl ether (1:1) modification of the procedure described by Ribí et al. (1962), (ii) the phenol extraction procedure (Westphal and Luderitz, 1954), and (iii) the trichloroacetic method of Boivin and Mesrobian (1935), as well as combinations of these treatments. In addition, 50% cell suspensions of *C. albicans* in 0.9% NaCl were treated by (iv) repeated freeze-thawing, both gradual and rapid; (v) autoclaving; (vi) heating for 1 hr at 100 C; (vii) treatment with 1.0% saponin combined with freeze-thawing; (viii) sonic treatment with a Bramson sonifier (30 kc) for 30 min; (ix) extraction with 1.0% NaOH; and (x) precipitation of extracts with varying volumes of ethanol. The extracted cells were always washed three to four times with 0.9% NaCl, and the washings were combined with the supernatant fractions which were dialyzed invariably against running tap water for 3 days. All treated cells were Gram-stained to ensure that the extraction procedures did not destroy the integrity of the yeast cell.

Immunological procedures. Agglutination studies were performed with untreated and all treated cells. All preparations were diluted to McFarland scale #4. All antisera produced, as well as the commercial anti-*Candida* serum (Difco) which served as control, were assayed and compared in this manner. Antisera absorbed with homologous and heterologous preparations were examined similarly. The double-diffusion

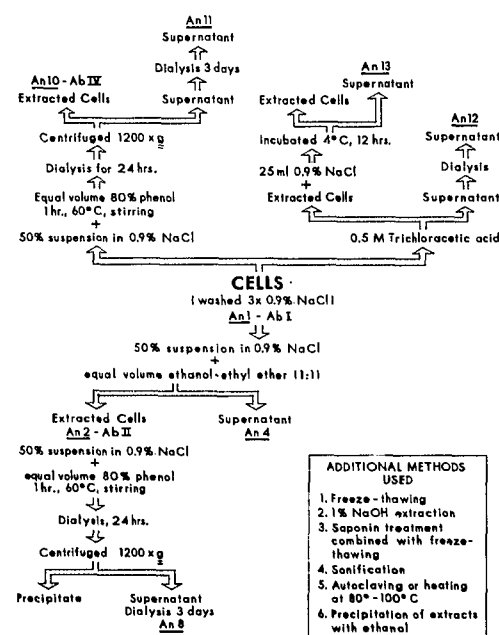


FIG. 1. Extraction methods applied to the variants of *Candida albicans*.

tube method and the plate agar gel diffusion method (Crowle, 1961) were applied to all antisera and the extracts.

Chemical analyses. All cells, extracts, and extracted cells were analyzed and standardized by determining the total nitrogen present by means of sulfuric acid digestion and nesslerization. Total carbohydrate was measured with the anthrone reaction. The presence of proteins or polypeptides was demonstrated by the ability of electrophoretically separated moieties to react with Poinceau-S (Allerhand et al., 1963). The ethanol-ethyl ether layer of that particular mode of extraction was examined for the presence of lipid material by gas chromatography through the courtesy of Robert Rosenfeld, Sloan-Kettering Institute, New York, N.Y.

Immunization. Rabbits weighing 2 to 3 kg were immunized by intravenous injections of standardized amounts of whole cells, extracts, and extracted cells three times per week over a 3-week period. The original dose was 0.5 ml; all subsequent intravenously administered doses were 1.0 ml. At 10 days after the last injection, animals were bled and challenged with an additional course of intravenous immunization when no

antibodies were demonstrable. Absence of antibodies in sera after the two courses of intravenous injections led to the use of antigens suspended in Freund's complete adjuvant. These injections were administered intramuscularly at 1-week intervals, and consisted of 2.0 ml of the adjuvant (Difco) emulsified just prior to injection with 2.0 ml of the material. This method was also employed when the necrotizing nature of some extracts obliterated the ear veins of the animals.

Virulence and toxicity tests. Lethal and LD₅₀ concentrations of the various strains were established by intravenous and intraperitoneal routes. When such values could not be obtained, mice were pretreated with cortisone (10 mg/kg). Toxicity of the various extracts was established by intravenous injection of the materials. Death within 12 hr was usual for the toxic materials. Animals receiving smaller doses or survivors were challenged 6 days after the first injection with lethal and toxic doses of the living organisms. The necrotizing ability of the extracts was determined by intradermal injections of 0.1 ml into rabbits and mice.

RESULTS

Virulence of the different strains. The LD₁₀₀ of strains V₁ and V₂ in mice was established at 10⁶ yeasts and the LD₅₀ at 10⁴ yeasts for the

intravenous and intraperitoneal routes, confirming the results of Hasenclever and Mitchell (1962a) for these strains. The toxicity of 10⁷ organisms of both mouse-virulent strains was also substantiated; mice died within 24 (usually 12) hr after the intravenous administration of such a dose. The avirulent strain, on the other hand, was not lethal to mice even when three doses of 10⁹ organisms were administered at 1-hr intervals. When the mice were pretreated for 2 weeks with daily injections of cortisone (10 mg/kg), rare fatalities occurred with 10⁹ avirulent yeasts introduced through the tail vein. Passage of *C. albicans* recovered from such animals through others similarly prepared did not lead to an increase in mouse-virulent properties.

Agglutination studies. Untreated cells, extracted cells, and the various aqueous extracts were used in the production of antisera. Only animals immunized with cells, regardless of treatment, displayed agglutinins. Antisera which resulted from immunization with whole cells reacted with homologous and heterologous cells (Table 1). It is quite obvious that the avirulent strain induces antibodies which react nonselectively with the heterologous virulent strains. On the other hand, the mouse-virulent yeasts induced antibodies which reacted more strongly with the virulent variants, whereas strain V₁ antisera manifested strain specificity. Agglutinins against whole cells in the control serum were of low reactivity with respect to three strains employed. Cells extracted with ethanol-ethyl ether, used as agglutinogens, were agglutinated to a slightly higher titer by the avirulent whole-cell antiserum. Variant V₁ agglutinins displayed enhanced strain specificity, especially with respect to the avirulent cells. Strain V₂ antiserum reacted more feebly with all cells extracted by this method, suggesting that the treatment removed required reactive sites from the cell surfaces of the immunizing antigen. Although phenol extraction of the cells did not affect the reactivity of the anti-A serum, appreciably increased agglutination titers were obtained with anti-V₁ and anti-V₂ sera. This extraction procedure apparently removed immunologically active V₁ strain-specific materials from the cells which seem required for the specificity of V₂ antibodies.

The difference in antibody-producing potentials between the avirulent and virulent strain became

TABLE 1. Agglutinins produced against untreated *Candida albicans*

Extraction	Variant	Agglutinins induced (AbI) against variant			
		A	V ₁	V ₂	Control (Difco anti- <i>Candida</i> serum)
None (An1)	A	640*	160	640	80
	V ₁	640	5120	2560	40
	V ₂	640	2560	2560	40
Ethanol-ethyl ether (An2)	A	1280	20	40	0
	V ₁	1280	2560	160	20
	V ₂	1280	1280	80	20
Phenol (An10)	A	1280	1280	2560	80
	V ₁	1280	10,240	640	80
	V ₂	1280	12,040	5120	40

* Numbers represent reciprocals of titers obtained with all antigens at density 4 (McFarland scale).

TABLE 2. Agglutinins produced against *Candida albicans* extracted with ethanol-ethyl ether

Agglutininogen		Agglutinins induced (AbII) against variant		
Extraction	Variant	A	V ₁	V ₂
None (An1)	A	640*	10,240	10,240
	V ₁	1280	10,240	10,240
	V ₂	1280	5120	10,240
Ethanol-ethyl ether (An2)	A	2560	10,240	10,240
	V ₁	1280	10,240	5120
	V ₂	1280	5120	10,240
Phenol (An10)	A	1280	10,240	10,240
	V ₁	640	10,240	10,240
	V ₂	640	10,240	10,240

* Numbers represent reciprocals of titers obtained with all antigens at density 4 (McFarland scale).

more manifest when antisera produced against cells extracted with ethanol-ethyl ether were employed against the same agglutinogens (Table 2). Strain specificity of anti-A antiserum (AbII) became manifest. Although untreated whole cells of the avirulent variety reacted with this serum to a lesser degree, agglutinogens of the homologous variety extracted with ethanol-ethyl ether (An2) and phenol (An10) were agglutinated to a higher titer. The antisera induced against An2 of the mouse-virulent variants displayed high titers without the specificity manifested by the antisera against An1. Thus, both antisera agglutinated A cells to the same degree as the homologous preparations; anti-V₁ serum (AbII-V₁) agglutinated An1 and An2 of the V₂ strain to a slightly lower titer, and anti-V₂ serum (AbII-V₂) acted similarly with An2 of the V₁ variety only.

Antisera produced against phenol-extracted cells reacted still differently when tested with the same agglutinogens (Table 3). Thus, the anti-A serum (AbIV-A) agglutinated with relatively good specificity the cells designated An1 and An10, but no specificity was displayed with regard to An2. The anti-V₁ serum (AbIV-V₁) displayed minimal specificity toward its homologous antigen in An2 preparations only, agglutinating An1-A to a higher titer. This property of reacting with the nonvirulent cells was shared

by the anti-V₂ serum (AbIV-V₂), except that it was manifested more readily with An10.

Agar diffusion studies. These studies were performed with the plate diffusion and the Full-throp modification of the tube diffusion methods (Crowle, 1961). The latter did not permit clear delineation of difference and, therefore, only the Ouchterlony studies are reported here. The antisera used in the agglutination studies were also employed for this method. The antigens, however, were dialyzed aqueous portions of extracts obtained by the various methods. These extracts failed to induce precipitins even after repeated courses of immunization and, since they could not stimulate agglutinins as mentioned before, they must be considered complex haptens. Figure 2 is a diagrammatic representation of the reactions observed when the various antisera were exposed to the ethanol-ethyl ether extract of whole cells (An4) and the same extract of cells previously treated with phenol (An4a). Anti-A serum produced against whole cells (AbI-A) did not react with any of these extracts. AbI-V₁, on the other hand, formed an identical precipitin band with all extracts except An4a-A, the ethanol-ethyl ether extract of avirulent cells extracted previously with phenol. A specific second precipitin band was obvious with the An4a-V₁ extracts. The corresponding anti-V₂ serum (AbI-V₂) precipitated these ethanol-ethyl ether preparations in a more complex pattern. A

TABLE 3. Agglutinins produced against phenol-extracted *Candida albicans*

Agglutininogen		Agglutinins induced (AbIV) against variant		
Extraction	Variant	A	V ₁	V ₂
None (An1)	A	5120*	10,240	10,240
	V ₁	1280	5120	10,240
	V ₂	1280	1280	10,240
Ethanol-ethyl ether (An2)	A	320	320	5120
	V ₁	1280	640	1280
	V ₂	320	320	1280
Phenol (An10)	A	5120	10,240	10,240
	V ₁	1280	10,240	10,240
	V ₂	1280	10,240	10,240

* Numbers represent reciprocals of titers obtained with all antigens at density 4 (McFarland scale).

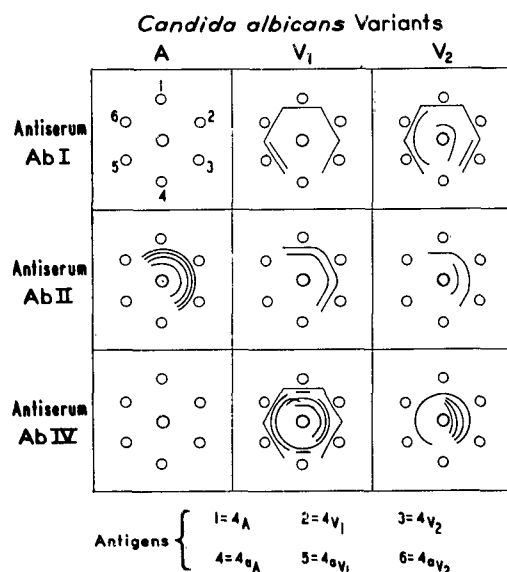


FIG. 2. Diagrammatic representation of the precipitin lines obtained with the various antisera and the complex haptens obtained by the ethanol-ethyl ether extraction of whole cells and cells previously extracted with phenol. Antigen wells 1, 2, and 3 contained 4_A, 4_{V1}, and 4_{V2}, respectively. Antigen wells 4, 5, and 6 contained the ethanol-ethyl ether extracts of cells previously extracted with phenol. The center well contained the antisera.

coalescent precipitin band was obvious about all of the complex haptens except the same An4a-A which failed to react with the anti-V₁ serum. A second pattern of fusion was formed with all of the directly obtained ethanol-ethyl ether extracts. A specific precipitin line was interspersed between the coalescent bands, formed with An4-V₂ which corresponds to the precipitin band observed with the phenol-ethanol ether extracts from the virulent strains (An4a). Reactions of the antisera produced against the cells extracted with ethanol-ethyl ether (AbII) were remarkable in that none of this group of antisera could react with the extracts produced by ethanol-ethyl ether extraction of cells pretreated with phenol (An4a). AbII-A reacted with An4 of all strains with four coalescent precipitin lines of a shape which strongly suggested that the extracts contain fractions of lower molecular weight than rabbit gamma-globulin. Two fused precipitin bands were formed by antiserum AbII-V₁, whereas only one identity precipitin line was formed by antiserum

AbII-V₂ with a single band formed in addition about extract An4-V₂. When antisera against the phenol-extracted cells (AbIV) were employed, no precipitin reaction was demonstrable again with the ethanol-ethyl ether extracts and the antiserum against the avirulent yeast. Antiserum AbIV-V₁ displayed one fusion precipitin line which excluded only the hapten 4a-A. A second coalescent band excluded extract An4-A but produced a pattern of intersection with a third looping band specific for only the ethanol-ethyl ether extracts. A third identity line could be discerned closest to the antibody well. The two extracts representing the avirulent strain were present with a precipitin line which appeared distinct for these complex haptens. It was interspersed between the outermost identity band and a coalescent line which is characteristic of An4a-V₁ and An4a-V₂. By comparison, the antiserum against phenol-extracted cells of the mouse-virulent *C. albicans* strain V₂ reacted with extracts An4 and An4a in a much simpler manner. A coalescent precipitin band was formed about all extracts except 4a-A. No further reactions were evident with the ethanol-ethyl ether extracts of phenol-extracted cells (An4a). However, a second looping line was formed about all three An4 extracts, and two additional identity bands formed about the ethanol-ethyl ether extracts of the virulent strains.

Figure 3 presents a summary of the precipitin reactions which occurred between the antisera and the dialyzed aqueous phenol extracts (An11) and the dialyzed aqueous phenol extract of cells extracted previously with ethanol-ethyl ether (An8). Antiserum AbI-A again did not react, whereas antiserum AbI-V₁ formed a single complete identity band with all extracts. An additional looping was evident about the complex haptens An8-V₁ and An8-V₂. The antiserum produced against untreated cells of the mouse-virulent yeast V₂ reacted with the phenol extracts of all variants but with a greater number of precipitin bands about the extracts from the virulent *Candida* strains. Three coalescent lines were formed about the An8 haptens with an additional line obvious with the extracts of mouse-virulent variants. Antisera produced against cells extracted with ethanol-ethyl ether resulted in patterns characteristic for each of the different organisms. Thus, the antiserum AbII-A elicited a pattern reminiscent of the one formed

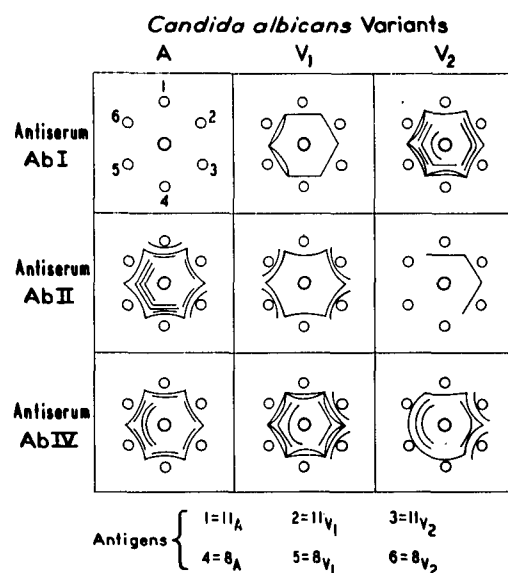


FIG. 3. Diagrammatic representation of the precipitin lines obtained with the various antisera and the complex haptens obtained by phenol extraction. Antigen wells 1, 2, and 3 contained the dialyzed aqueous portions of the phenol extracts of the avirulent and the virulent variants; the remaining three wells held the extracts obtained by the same method applied to the three strains after prior extraction with ethanol-ethyl ether. The antisera were deposited in the center well.

with antiserum AbI-V₁ with the same extracts. This was, however, the first time that an arc very close to the antigen wells became evident, and will be discussed later on. The pattern which emerged with antiserum AbII-V₁ was a single coalescent band with a short precipitin arc about the antigen wells of the avirulent strains. Antiserum AbII-V₂ reacted only with the direct phenolic complex haptens, forming a single looping. The precipitin patterns formed with the antisera produced as a result of immunization with phenol-extracted cells did not differ so strikingly from one another. Differences were, however, still readily discernible. Antiserum AbIV-V₂ was less complex and presented virulent strain-specific bands. The specific reactions of the other virulent yeast were more pronounced; even the antiserum against the avirulent variety (AbIV-A) displayed the capacity to form a special precipitin line with the complex haptens An8-V₁ and An8-V₂. Figure 4 represents the

reactions of the same antisera with the extracts obtained by trichloroacetic acid treatment. Apparently only a single moiety was removed in this manner. The moiety diffused very slowly, resulting in a precipitin arc very close to the antigen depression, and resembled and may be identical with the similar configurations described before. It is interesting that all three sera produced against the cells treated with ethanol-ethyl ether displayed this precipitation pattern, whereas only the antisera against the whole cells of the virulent strains precipitated the extracts. The antisera against phenol-treated cells crossed the line of mouse virulence. AbIV-A and AbIV-V₁ precipitated the trichloroacetic acid extract only.

Treatment of the extracts with 6 volumes of ice-cold ethanol resulted in precipitates of which only the phenol extract precipitates displayed the ability to react with the antisera. The antisera produced against the phenol-treated cells reacted differently with the precipitates than with the original extracts (Fig. 5). Thus, antiserum AbIV-A showed a coalescent band, but the areas before the precipitate-containing depres-

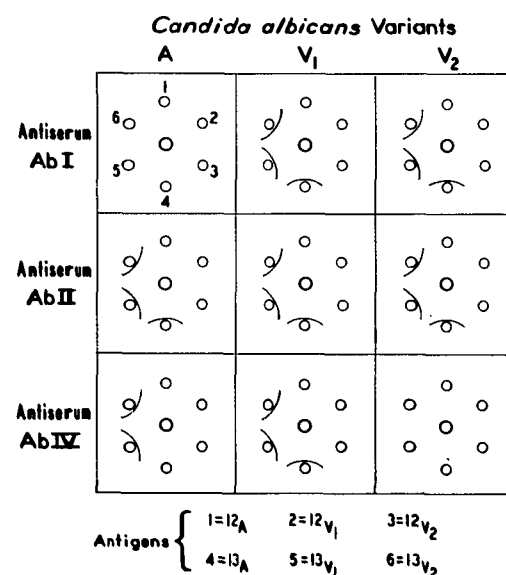


FIG. 4. Diagrammatic representation of the precipitin lines obtained with the various antisera and the trichloroacetic acid extracts in wells 1, 2, and 3 and the 0.9% NaCl washings of the trichloroacetic acid-extracted cells in wells 4, 5, and 6. The center wells held the antisera.

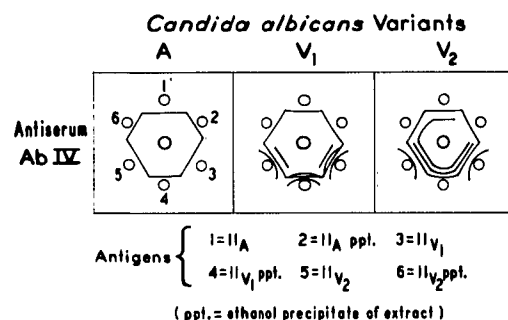


FIG. 5. Diagrammatic representation of the comparison between the reactions between antiserum AbIV of the three strains and the phenol extracts and the ethanol precipitates of the same extracts.

sions were shorter. The precipitin lines formed with antiserum AbIV-V₁ and the precipitates indicate that one of the complex haptens of strain V₁ and two of strain V₂ capable of reacting with antiserum AbIV-V₁ were not precipitated by ethanol. Missing precipitin arcs were also evident with antiserum AbIV-V₂. The shorter arcs in front of the precipitated complex haptens described with the antiserum against the avirulent yeast were also demonstrated with antisera against the virulent varieties, suggesting some alteration in the precipitated haptens which does not affect their ability to react with the antisera.

To appreciate the topographical distribution of the complex haptens, antisera against extracted cells were absorbed with homologous and heterologous whole cells (Table 4). The ability of the whole cell to remove specific antibody from the antisera produced against treated cells appears quite limited, suggesting that the treatment removes, to a considerable degree, the materials which constitute the complex haptens capable of reacting and bares different sites on the cell surface which are themselves antigenic. In a few instances, an additional precipitin line appeared after absorption with whole cells, suggesting the removal of a reaction inhibitor by the intact cells.

Chemical composition of extracts. The chemical analyses were intended to follow the major postulated constituents of *Candida* cell walls (Salton, 1960). The findings (Table 5) score the predominance of the carbohydrate constituents, especially when phenol extraction was applied. The quantitative difference between the mouse-

avirulent yeasts is quite apparent. Ethanol-ethyl ether extracts examined by electrophoresis and immunoelectrophoresis displayed several bands which reacted with immune sera but also stained readily with Poinceau-S, suggesting the presence of proteins at least as part of the antigenic make-up of *Candida*. It was usual to encounter a viscous white layer at the water-ethyl ether interface during the ethanol-ethyl ether extraction of the mouse-avirulent yeast. Chemical examina-

TABLE 4. Effect of absorption with whole cells on the precipitin-forming ability of anti-*Candida* serum AbII*

Antiserum (AbII) against strain	Untreated cells used for absorption	Extracts					
		4-A	11-A	4-V ₁	11-V ₁	4-V ₂	11-V ₂
A	Control	2	1	2	1	2	3
	A	0	0	0	0	2	0
	V ₁	0	±	3	0	3	0
	V ₂	0	0	2	0	2	0
V ₁	Control	0	0	1	1	2	±
	V ₁	0	0	1	1	2	0
	V ₂	0	0	0	±	2	0
V ₂	Control	0	0	3	0	2	0
	A	0	0	3	0	3	0
	V ₁	0	0	3	0	3	0
	V ₂	0	0	2	0	2	0

* The numerals refer to the number of precipitin bands. Antisera against the ethanol-ethyl ether extracted cells of each variant were each exposed to packed untreated cells of the homologous and heterologous strains (0.75:0.25), incubated at 37 C for 2 hr and 4 C for 24 hr, centrifuged at 1200 × g, and the supernatants were used. Serum diluted with 0.9% NaCl (0.75:0.25) served as control.

TABLE 5. Carbohydrate-nitrogen ratios of the complex haptens*

Variant	Ethanol-ethyl ether			Phenol		
	1	2	1:2	1	2	1:2
A	30	3.32	9	14.6	3.2	4.56
V ₁	53	7.22	7.5	91.0	1.5	60.0
V ₂	78.5	9.6	8.2	96.0	0	—

* Symbols: 1 = mg of carbohydrate per 100 mg of dry extract; 2 = mg of nitrogen per 100 mg of dry extract.

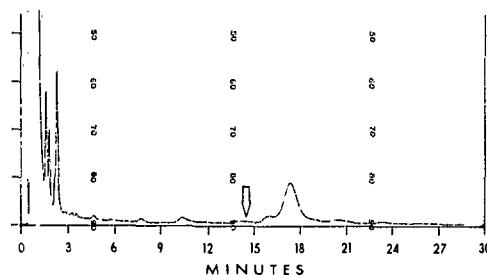


FIG. 6. Gas chromatogram obtained with the material concentrated at the water-ethyl ether interface during the ethanol-ethyl ether extraction of the mouse-avirulent strain. The peaks obtained from 1 to 3 min represent fatty acids; those about 18 min correspond to phytosterols. The arrow indicates the usual position of cholesterol.

tion of this material showed no carbohydrate or nitrogen, but fatty acids and plantlike sterols were observed by gas chromatography. Calcium was also present (Fig. 6).

Toxic properties of the extracts. The endotoxin-like nature of the complex haptens extracted from the mouse-virulent strains became apparent clinically during the immunization procedures, as reported cursorily earlier (Isenberg, Allerhand, and Berkman, 1963). Rabbits which received intravenous preparations of both ethanol-ethyl ether and phenol extracts manifested extensive necrosis of the skin around the sites of injection after the second dose. Similar severe skin reactions were observed when mice were treated with the same materials. When 0.2 ml of the phenol extracts of the mouse-virulent strains were injected into the tail veins of mice, 50% died within 12 hr. The survivors did not succumb when challenged intravenously with 10^7 cells of the homologous strain, a uniformly toxic and lethal dose in unprotected controls. Corresponding doses of the ethanol-ethyl ether extracts were not lethal to mice, but all animals so treated were severely ill for at least 2 days, and subsequent challenge with the number of yeasts fatal to controls failed to kill. The mouse-avirulent strain did not elicit any response in mice, not even those pretreated with cortisone. Extracts or extracted cells produced no ill effects. It is of great interest, however, that 25% of the animals pretreated with the ethanol-ethyl ether extract of the avirulent variety succumbed to challenge with 10^7 cells of the homologous strain. This observation was repeated several times but

requires further study before an adequate explanation can be advanced.

DISCUSSION

The findings presented here permit comparison between the immunological and chemical studies performed with yeast and especially *Candida* (Jonsen, Rasch, and Strand, 1955; Jonsen, 1955; Bishop, Blank, and Gardner, 1960) and the observations that virulence of this yeast is associated with a toxic principle (Salvin, 1952; Mourad and Friedman, 1961; Hasenclever and Mitchell, 1962a, b; Roth and Murphy, 1957). The difference between the mouse-avirulent and the mouse-virulent variants is obvious from the immunological reactions with agglutinations, precipitations, and antibody-absorption studies. These differences are quite evident chemically and are finally confirmed by biological activities. Although differences between the two virulent variants exist, they appear to share a common toxic or virulence-enhancing cell-wall constituent. The chance that this moiety may be found near or on the cell surface is suggested by the behavior of antisera formed against the whole cells which react with the various extracts. The same antiserum against the avirulent variant (AbI-A) fails to form precipitin lines, indicating a lack of functional complex haptens involved with the production of a toxic effect in the living host. The reaction of the agglutinogens in the same sera may be used as further proof of this topographic difference between the virulent and avirulent strains. The quantitative chemical differences between these strains cannot as yet be assigned to a definite locus on the cell surface. However, the mild treatment with ethanol-ethyl ether liberates reactive sites on the avirulent strain, which are removed by pretreatment with phenol as demonstrated by the precipitin reactions between antiserum AbII-A and the extracts An4 and An4a. Since this extraction results in the liberation of copious lipid material from the avirulent *C. albicans*, one is tempted to postulate that this lipid material interferes with the building up of the reactive sites among which one finds the toxic elements. This, of course, suggests a metabolic difference, for the reduced amount of carbohydrate material in this avirulent strain is apparently combined with or even hidden by the much increased lipid. The findings of Bishop et al. (1960) would make one suspect that the

active hapten must be a glucan (characterized by being a highly branched polymer containing a preponderance of β 1-6 linkages in addition to β 1-3 linkages) or a mannan (composed of highly branched relatively short chains of α 1-2 linked mannose unit joined together by α 1-6 linkages), rather than chitin, which these authors also reported. The latter could not be part of the highly toxic phenol extract of strain V₂, which contained no nitrogen. When their extraction procedure was applied to the three strains of yeasts in experiments not included in this report, several additional immunologically active fractions were included, suggesting that this treatment liberates additional substances not necessarily located in the vicinity of the cell surface. The toxic reaction elicited by the extracts has been described as endotoxin-like advisedly, since the classic endotoxins are by definition confined to the somatic antigens of gram-negative bacteria, and their toxicity is defined by many additional biological criteria which have not as yet been applied to these extracts. The studies of Ribi et al. (1962) led to the hypothesis that a macromolecular complex of critical size is one of the major requirements for endotoxin to elicit its characteristic effects in the mammalian host. One is tempted to assume that, although polysaccharides of *C. albicans* may differ in many respects from the somatic antigens of the gram-negative bacteria, they may produce large enough polysaccharides to warrant their designation as endotoxin-like materials if not as endotoxins.

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